

**THE HUMAN SKELETAL MUSCLE IN VIVO –
THE USE OF MICRODIALYSIS TO STUDY
GLUCOSE METABOLISM AND INSULIN
RESISTANCE**

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ABSTRACT

The aim of this thesis was to further develop the microdialysis technique for studies of glucose metabolism and insulin resistance in human skeletal muscle in vivo and to use this technique to explore the effect of exercise on muscle glucose metabolism and insulin action. The first two studies were conducted to explore the possibility of using a perfusion flow rate low enough to obtain complete equilibrium between the interstitial and perfusion fluids and if the colloid included in the perfusion fluid for this purpose affected the metabolite concentrations in the dialysate. The following three studies focus on glucose metabolism and insulin resistance by examining the extent to which insulin and/or the insulin-mimetic trace element vanadate added to the perfusion fluid exerted local effects on the glucose metabolism in healthy and in insulin-resistant human skeletal muscle, at rest and following a single bout of exercise.

It was found that to obtain complete equilibration between the perfusion fluid and the interstitial fluid in human skeletal muscle, a perfusion flow rate as low as 0.16 $\mu\text{l}/\text{min}$ is necessary with the presently used catheters (Study I).

At these low perfusion flow rates a colloid needs to be included in the perfusion fluid in order to keep the fluid balance and in this thesis it was shown that the colloid dextran-70 does not affect the metabolite concentrations in the collected dialysates (Study II).

A high cut-off microdialysis membrane can be used to infuse insulin into human skeletal muscle and to record its effect on metabolite concentrations (Study III).

When the insulin-mimetic agent vanadate was infused into human skeletal muscle (Study IV), it was found to decrease the interstitial glucose concentration in a similar way as insulin, but in contrast to insulin, the effect of vanadate was not diminished in insulin-resistant skeletal muscle and was not affected by exercise (Study V).

In insulin-resistant human skeletal muscle, the effect of insulin tended to be larger following a single 2-h bout of exercise than in resting muscle (-32 % versus -20%, $p < 0.09$) (Study V). The insulin effect in resting muscle of healthy individuals was -30 % (Study III).

In conclusion, the studies in the present thesis support the hypothesis that it will be possible in the future to measure insulin resistance in skeletal muscle with microdialysis and to use the technique to explore the effect on skeletal muscle of different therapeutic options in the treatment of type-2 diabetes. On this line, the present results indicate that a single bout of exercise normalizes the insulin effect on the interstitial glucose concentration in insulin-resistant human skeletal muscle.

Key words: microdialysis, human skeletal muscle, glucose, lactate, pyruvate, glycerol, urea, dextran, vanadate, one-legged exercise

LIST OF PUBLICATIONS

The present thesis is a summary and discussion of the results presented in the following papers, which will be referred to in the text by their Roman numerals.

- I. Rosdahl H. Hamrin K. Ungerstedt U. Henriksson J. Metabolite levels in human skeletal muscle and adipose tissue studied with microdialysis at low perfusion flow. *Am. J. Physiol.* 274:E936-E945, 1998.
- II. Hamrin K. Rosdahl H. Ungerstedt U. Henriksson J. Microdialysis in human skeletal muscle: effects of adding a colloid to the perfusate. *J Appl Physiol.* 92: 385-393, 2002.
- III. Rosdahl H. Hamrin K. Ungerstedt U. Henriksson J. A microdialysis method for the in situ investigation of the action of large peptide molecules in human skeletal muscle: detection of local metabolic effects of insulin. *Int J Biol Macromolecules.* 28(1):69-73, 2000.
- IV. Hamrin K. Henriksson J. Local effect of vanadate to increase glucose uptake and lactate formation in human skeletal muscle. *Life Sciences.* In press.
- V. Hamrin K. Henriksson J. Local effect of insulin and vanadate in insulin-resistant human skeletal muscle after one bout of one-legged exercise. Manuscript.

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LIST OF ABBREVIATIONS

ANOVA	analysis of variance
ADP	adenosine diphosphate
AMP	adenosine monophosphate
ATP	adenosine triphosphate
cut-off	the maximal weight (Da) of molecules that can diffuse across the dialysis membrane
FFA	free fatty acids
GLUT 4	glucose transporter 4
i.d.	inner diameter
KHB	Krebs-Henseleit buffer
mol. wt.	molecular weight
NADH	nicotinamide adenine dinucleotide, reduced form
o.d.	outer diameter
OGTT	oral glucose tolerance test
SD	standard deviation
SEM	standard error of the mean
TNF- α	tumor necrosis factor- α

INTRODUCTION

Through the degradation of carbohydrates, proteins and lipids, energy is released and available to use, e.g. in a muscle contraction or for synthesis of compounds. The energy that is not used at once is stored, mainly as triglycerides in the adipose tissue (Cahill, 1970) or as glycogen in the skeletal muscle and in the liver (Felig, 1976; Hultman, 1971). The energy liberating reactions are often coupled to the energy demanding ones. The interplay between those processes is what we call metabolism. Energy homeostasis is achieved when the metabolism is in balance.

SKELETAL MUSCLE METABOLISM

Cells in human skeletal muscle use, as most cells, ATP as energy source and generate ATP mostly from oxidation of glucose and free fatty acids (FFA). When glucose enters the cell it is either stored as glycogen or undergoes glycolysis. The fate of glucose is determined by the energy demand on the cell. Glucose initially stored as glycogen can later undergo glycogenolysis/glycolysis. Pyruvate, the end product of glycolysis, can then be oxidized aerobically via the Krebs cycle or converted to lactate. The glycolysis net yield of energy is 2 moles of ATP per mole of glucose (or 3 if the glucose is derived from cell glycogen) and the Krebs cycle net yield is 36 moles ATP/mole glucose. The energy yield from FFAs varies with its size. The most abundant fatty acid in the body, palmitate, yields 129 ATP (Devlin, 1998).

Glucose entry into cells is not a process of simple diffusion; it requires the involvement of a carrier protein in order to facilitate the movement of glucose across the plasma membrane. In skeletal muscle cells the predominant carrier protein is GLUT4. If the skeletal muscle cell is stimulated by insulin or exercise, GLUT4 is translocated from intracellular pools to the plasma membrane and thus facilitates glucose to enter the cell.

MICRODIALYSIS

Background

The history of microdialysis goes back to the early work of Bito et al (Bito, 1966) and Delgado et al (Delgado, 1972). Ungerstedt and Pycoc (Ungerstedt, 1974) refined and developed the microdialysis sampling technique in vivo in brain tissue. From the early applications in monitoring neurochemical events in animals, the use of microdialysis has been spread to many other areas of research including measurements in adipose tissue (Arner, 1990; Lönnroth, 1987), blood (Arner, 1988), ocular tissues (Ben-Nun, 1989; Waga, 1991), spinal cord (Backström, 2001), pancreas (Stock, 1990), skeletal muscle (Henriksson, 1990), tumors (Palsmeier, 1994), myocardium (Kuzmin, 1992; Zemgulis, 2001), skin (Anderson, 1992; Petersen, 1992), liver (Scott, 1993), spleen (Shimizu, 1994), bone (Stolle, 2003; Thorsen, 1996), intestine (Oldner, 1996; Tenhunen, 1999), tendon (Langberg, 1999), lungs (Herkner, 2002; Oldner, 1999) and joints (Lawand, 2000).

Important reasons for the attractiveness of microdialysis are the atraumatic character as well as the possibility to sample from the interstitium rather than from the often less relevant blood compartment (Bolinder, 1993; Lönnroth, 1987). Another advantage with

microdialysis is that it is possible to analyze the sample immediately, without further purification. This is due to the fact that only low molecular weight substances pass the membrane, thus the sample is clean and can be used in most assays without further treatment. In addition, biologic compounds in the sample are protected from enzymes which cannot pass the microdialysis membrane. Microdialysis has also been used in several clinical studies (reviewed in Klaus, 2004).

Principle of microdialysis

The basic principle of microdialysis is to mimic the passive function of a capillary blood vessel. The microdialysis device consists of a catheter which in the distal part has a tubular dialysis membrane permeable to water and small solutes. The catheter is inserted into the tissue and continuously perfused with a solution that resembles the interstitial fluid. Through connective tubing, perfusion fluid is pumped through the catheter with a perfusion pump. The perfusion fluid that has passed the membrane, named dialysate, is collected at the end of the outlet tubing in a microvial and analyzed chemically. When the perfusion fluid passes the membrane it equilibrates with the fluid outside the membrane by diffusion in both directions. The composition of the dialysate therefore reflects the composition of the interstitial fluid of the tissue being sampled.

Calibration

When microdialysis is not performed under equilibrium conditions, the dialysate concentrations are only a fraction of the interstitial concentration. In this case calibration is a prerequisite. The ratio between the concentration of a particular substance in the dialysate and the interstitial concentration of the same substance is defined as *the relative recovery* in microdialysis experiments. The calibration procedure aims at finding out the relative recovery of the metabolite of interest.

Recovery

In addition to relative recovery, absolute recovery sometimes needs to be considered in microdialysis experiments. Absolute recovery is defined as the total amount of the substance harvested in the outflow per unit time. If the concentration of a substance outside the catheter is increased, more of the substance will diffuse over to the perfusate since the concentration gradient is changed. This results in an unchanged relative recovery but an increased absolute recovery. Relative recovery can be considered to be constant as long as the diffusion conditions are the same, while the absolute recovery varies with the interstitial concentration of the substance. The recovery is influenced by various factors:

Perfusion flow rate

Absolute recovery decreases and relative recovery increases non-linearly when perfusion flow decreases (Benveniste, 1989). With very low perfusion flow rates, the relative recovery may approach 100 % (Alexander, 1988; Menacherry, 1992). In this thesis we show that with very low perfusion flow rate that it is possible to reach full equilibration between interstitial and perfusion fluids. The relative recovery is then 100 %.

Area of the dialysis membrane

Both absolute and relative recovery increases proportionally when the effective surface area of the dialysis membrane is increased (Benveniste, 1989). When designing a microdialysis experiment, the perfusion flow rate and the membrane area are the two possible ways of influencing the relative recovery. Usually the limiting factor is the sample volume required for analyzing the metabolite of interest. A low perfusion flow rate gives a high relative recovery and low absolute recovery, thus minimizing the amount of drainage of substances from the tissue, but requires longer collection times.

Speed of diffusion within the tissue

Diffusion in an inhomogeneous tissue is impeded by cell membranes which will give rise to a prolongation of the diffusion pathway (compared to the in vitro situation). This is probably the major rate-limiting factor in microdialysis measurements rather than the passage through the dialysis membrane (Amberg, 1989). Diffusion is also dependent on the radius of the solute. The diffusion coefficient is inversely proportional to the radius and hence also to the molecular weight of the solute (Benveniste, 1989).

Properties of the dialysis membrane

Certain substances may adhere to or interact with the membrane via unknown mechanisms and this affects the recovery (Benveniste, 1989). The microdialysis membranes are available with different molecular weight cut-offs and this obviously determines the size of the substance that can be recovered.

Tissue temperature

The relative recovery is directly related to the temperature of the tissue. The diffusion coefficient is increased at a higher temperature (Benveniste, 1989). This should especially be taken into consideration when in vitro recovery is determined. In the in vivo situation a perfusate solution at room temperature (instead of body temperature) does not seem to affect metabolite concentration as long as the solution is not hypotonic (de Lange, 1994).

No-net flux method

One method to estimate the in vivo recovery is the no-net-flux method, introduced by Lönnroth et al (Lönnroth, 1987). The procedure involves perfusing the same compound at several different concentrations through the microdialysis catheter when it is inserted in the tissue. Selected concentrations should be above and below the expected interstitial concentrations. The difference between the concentrations in dialysate and perfusate is plotted in a graph against the concentration of the compound in the perfusion medium. Using linear regression it is possible to calculate the point where no diffusion occurs (no-net-flux). At this point, when the concentrations in dialysate and perfusate are equal, the concentration in the perfusion medium is the same as the interstitial concentration. The slope of the regression line provides the recovery. Interstitial concentration has to be stable during calibration and the recovery assumed not to change during the experiment. The method is very time consuming and therefore most suitable as a reference method when evaluating other calibration techniques.

Zero-flow method

Another calibration method is the zero-flow method (Bungay, 1990; Ekblom, 1992; Jacobson, 1985). The perfusion flow rate is varied and the different dialysate concentrations are plotted, then using non-linear regression analysis to extrapolate to zero flow, the recovery is determined. At zero flow the relative recovery will be 100 %. This method has about the same advantages and disadvantages as the no-net-flux method although the zero-flow method is more mathematically demanding.

Retrodialysis

The method of retrodialysis or internal reference technique (Jansson, 1994; Larsson, 1991; Scheller, 1991) consists of adding a marker to the perfusate. The marker should be a compound with diffusion characteristics close to the substance of interest, most commonly it has been a labeled analogue. The recovery in vivo can be achieved after measuring the relative loss of a substance diffusing from the perfusate to the interstitial fluid. It is assumed that the flux over the membrane is equal in both directions and that the marker has similar diffusion properties as the substance sampled from the interstitial fluid. A major advantage with this method is that the recovery can be measured continuously and at the same time as the experiment.

Near-equilibrium

When the perfusion flow rate is very low and the membrane is long, the recovery is assumed to be near 100 % (Alexander, 1988; Bolinder, 1992; Hagström-Toft, 1997; Menacherry, 1992; Moberg, 1997). The concentrations on both sides of the membrane then have time to equilibrate totally. No calculations are needed and the true interstitial concentration is measured directly. The amount of sample obtained is relatively small though and therefore requires relatively long sampling periods. A positive feature with this technique is that the absolute amount of substances drained from the tissue is kept at a minimum. When microdialysis is performed at high flow rates, a concentration gradient can develop which depletes the interstitial compartment of the compound of interest.

The near-equilibrium technique seemed to be ideal for our experiments. The papers included in this thesis are based on that method.

Colloid

To make sampling at low perfusion flows possible in skeletal muscle, without a substantial loss of perfusate into the tissue, a colloid needs to be included in the perfusion fluid (Rosdahl, 1997). In the referred study it was shown that when dextran-70 at 40 g/L is used, no net loss of perfusate occurs, even if the perfusion flow is as low as 0.16 $\mu\text{L}/\text{min}$ (Rosdahl, 1997). When the membrane of the microdialysis catheter has a higher molecular cut-off (larger pore-size), the use of a colloid or a push-pull pumping setup is necessary even at higher perfusion flow rates to prevent fluid loss (Hoffner, 2003; Sjögren, 2002). If the interstitial pressure decreases in the tissue there will also be a fluid loss if the perfusion fluid is not supplemented with a colloid (Langberg, 1999).

Microdialysis in the human skeletal muscle

It was reported in 1993 that microdialysis can be used successfully in human skeletal muscle (Rosdahl, 1993). Since then microdialysis in human skeletal muscle has been used to measure absolute concentrations of glucose, lactate, glycerol, urea, pyruvate, thromboxane, amino acids, insulin, prostaglandin, adenosine, AMP, ADP, ATP, purines, choline, potassium, glutathione vascular endothelial growth factor (VEGF), creatine, neuropeptide Y and calcium (Ernberg, 2004; Frandsen, 2000; Green, 1999; Gutierrez, 1999; Hagström-Toft, 1997; Hellsten, 1998; Hoffner, 2003; Korth, 2000; Lundberg, 2000; Müller, 1995; Persky, 2003; Samii, 2004; Sjöstrand, 1999; Tonkonogi, 2003) as well as blood flow (Hickner, 1994). Most experiments have been performed in resting skeletal muscle but in some investigations static (Green, 1999; Rosdahl, 1993) or dynamic muscle contractions (Hellsten, 1998; Juel, 2000)(Reviewed in (Bangsbo, 1999) were also studied.

Effect of catheter insertion

The microdialysis technique involves the introduction of a foreign material into a tissue. The response to implantation of a microdialysis catheter can broadly be classified as; trauma caused by guide or catheter insertion, effects on the vascular system over a wider area and inflammatory reactions to the catheter (Anderson, 1994). Most studies investigating the effect of catheter insertion have been performed in rat brain or human skin. In the rat brain there are changes in both local glucose metabolism and blood flow within three hours following insertion. These changes are not seen 24 hours after insertion though (Benveniste, 1989). Signs of lesions in the blood-brain barrier in the rat brain undergoing catheter insertion are seen 10 – 30 min following implantation, but not thereafter. These effects may be due to changes that are specifically associated to the brain metabolism and does not necessarily have to occur in skeletal muscle. Around catheters inserted into human skin investigators have seen an increased blood flow immediately after insertion, which had nearly normalized after 60 min (Anderson, 1994). Groth et al (Groth, 1998) have suggested that an equilibration period of 90 – 120 min is required after catheter insertion in human skin. To reduce the effect of insertion, local anesthesia is furthermore suggested prior to insertion (Anderson, 1994). Histological examination of rat skeletal muscle at 1, 6, 12 and 32 hours after catheter insertion reveals very little damage. No pocket of extra cellular fluid and no evidence of bleeding were found. Infiltration of lymphocytes could be seen beginning at six hours and continued over the next 24 hours but no morphological changes were seen and the lymphocyte infiltration did not affect the recovery over the dialysis membrane (Palsmeier, 1994). Levels of glutathione were high immediately after implantation in rat skeletal muscle and declined to steady state levels after 90 min (Sirsjö, 1996). The insertion of a catheter in human skeletal muscle results in leakage of glycerol from muscle cells to the interstitial space causing a rise in interstitial concentration of glycerol. High glycerol concentration and elevated amino acid concentrations in human skeletal muscle caused by cellular damage imply that a period of rest of at least 90 – 150 min from insertion of the catheter is required before baseline collection starts (Gutierrez, 1999).

INSULIN SENSITIVITY

Plasma glucose is tightly regulated around an average concentration of 4.5 mM in healthy humans. When the concentration falls below 3.3 mM, central nervous system function becomes progressively impaired and death may ensue (Berne, 1993). A high concentration of glucose in the blood is a characteristic feature of diabetes mellitus and can also be a risk factor for a number of complications including cardiovascular diseases, cerebrovascular diseases and neoplasms (Balkau, 1998). The key hormone regulating blood glucose levels is insulin. Insulin affects almost every type of cellular function ranging from acute metabolic effects, including glucose transport and inhibition of lipolysis, to long term cell growth and division. Regarding blood glucose levels, skeletal muscle is one of three organs that, above all, respond to insulin in a way that markedly influences the blood glucose concentration. Of these organs skeletal muscle is quantitatively the largest tissue involved in maintaining glucose homeostasis (DeFronzo, 1988). The skeletal muscle is also able to take up glucose in response to exercise (contractile activity) without involving insulin. An exercise session therefore increases the glucose transport activity specifically to the active tissue and this is associated with a transient decline in circulating insulin levels during endurance exercise (Short, 1997). In exercised trained people the circulating levels of insulin are furthermore decreased at rest in response to e.g. oral glucose (Mikines, 1989). Exercise trained people therefore need less insulin in order to maintain glucose homeostasis—their insulin sensitivity is increased. The insulin sensitivity increases with increasing amount of physical activity. This increased sensitivity applies especially to the skeletal muscles involved in the exercise (Mikines, 1989). This may be one factor explaining why physical activity is one of the best methods to decrease the risk of developing cardiovascular diseases.

Insulin resistance

The opposite of insulin sensitivity is insulin resistance and this can be defined as a condition where insulin is unable to exert a normal biological response in its target tissues. Insulin resistance is a well known risk factor in cardiovascular disease and together with abdominal obesity, is often referred to as the metabolic syndrome or insulin resistance syndrome (Reaven, 1988). Insulin resistance is also a prominent feature of individuals with Type II diabetes mellitus. Development of insulin resistance in skeletal muscle precedes the development of Type II diabetes mellitus (Vaag, 1992). The pathophysiological mechanisms of insulin resistance are poorly understood. There are a number of sites in the body where the resistance can occur; in the pancreas, from where the insulin is released, somewhere along the route of transport to the tissues (e.g. the capillary wall), in the liver (which then liberates too much glucose), the muscle or fat cell membrane (e.g. lack of receptors) or inside the muscle or fat cell (abnormal signal transduction). Also overproduction of FFAs from the adipose tissue and adipocyte overexpression of tumor necrosis factor- α (TNF- α) have been implicated to cause insulin resistance (Hotamisligil, 1995; Shulman, 1999). The insulin resistance causes an increased insulin secretion from the β -cells in the pancreas resulting in hyperinsulinemia. This means that the abnormality in insulin-resistant states can be due to the insulin resistance itself or chronic effects of compensatory hyperinsulinemia (Ferrannini, 1998).

Measuring insulin resistance in vivo

Since insulin resistance is one of the earliest signs of cardiovascular risk and Type II diabetes mellitus there is a need for an accurate and reproducible method for measuring insulin resistance in vivo. At a whole body level, based on peripheral blood sampling, any insulin effect is a compound effect induced in various tissues. This is probably the reason why several techniques to measure insulin resistance in vivo have been devised. There is a general agreement that the glucose clamp technique (DeFronzo, 1979) is the golden standard for measurement of insulin action. With this technique insulin is infused at a constant preset rate and the plasma glucose concentration is clamped at the normal fasting (5 mM, euglycemic) or any pre-existing (isoglycemic) level. The glucose concentration in plasma is held constant via glucose infusion through an adjustable pump and blood is drawn at regular intervals (normally every fifth minute) to measure plasma glucose concentration. When a steady state is attained, the glucose infusion rate equals the amount of glucose disposed of by all the tissues in the body which is a measure of overall insulin sensitivity.

A more indirect method is the oral glucose tolerance test (OGTT). The technique is much less demanding than the glucose clamp technique; it provides information about insulin secretion and action but does not directly yield a measure of insulin sensitivity. The subject drinks a solution of 75 g glucose mixed in (250 ml) water. Blood samples are collected before drinking the solution and thereafter every 30 min for two to three hours for the measurement of plasma glucose and insulin. This test is widely used in the clinic to diagnose Type II diabetes. The diagnostic criteria for diabetes is a fasting blood (venous) glucose > 6.1 mM and/or a 2-hours post load concentration of >10.0 mM (WHO, 1999). Impaired glucose tolerance is said to occur if the 2-hours post load concentration is > 6.7 mM (WHO, 1999).

All methods available today to measure insulin resistance determine the whole body insulin resistance. A method to measure the local insulin resistance in a single tissue would be interesting in evaluating the origin of insulin resistance. The hypothesis behind the studies in this thesis is that it will be possible in the future to measure insulin resistance with microdialysis.

Insulin adsorption of plastic tubing

When handling insulin in a solution it is important to be aware that insulin is known to be adsorbed by different materials such as glass and polyacrylamide (Petty, 1974). It seems as if the adsorption varies inversely with the concentration of insulin in the solution (Hirsch, 1977). It is also known that albumin may have a protecting effect in reducing the insulin adsorption (Schildt, 1978). This is the reason why a small amount of the patient's own blood is added to the insulin solution when performing a glucose clamp experiment (DeFronzo, 1979).

The extent of adsorption to tubing is determined by a drug's diffusion coefficient, the length of tubing and the solution flow rate (Elliott, 1980). The amount of insulin adsorption to the membrane of a microdialysis catheter has not, to our knowledge, been examined. This should therefore be investigated more thoroughly before continuing examinations of giving insulin via the perfusion solution of a microdialysis catheter.

VANADATE

Vanadium is a trace element that occurs in concentrations ranging from 0.1 to 3 nmol/g (Post, 1979) in most mammalian cells. The physiological role of vanadium is not known, but it seems to be needed for normal growth and development (Nechay, 1984). Vanadate, the salt of vanadium, which resembles phosphate structurally, has in recent years been demonstrated to mimic most of the biological effects of insulin in several cell types (Shechter, 1990). This makes vanadate a potential therapeutic tool of clinical interest in diabetes mellitus type-2.

In skeletal muscle most studies report that vanadate stimulates the glucose uptake through a different signaling pathway than insulin. Available data indicate that this pathway resembles that of the hypoxia-induced glucose transport (Cortright, 1997). In human skeletal muscle *in vitro*, vanadate stimulates glucose transport both in insulin-resistant (Carey, 1995) and insulin-sensitive muscle (Cortright, 1997). However, concerning the function of vanadate in human skeletal muscle *in vivo*, no information is available.

AIMS

Microdialysis gives a unique possibility to study metabolic events in the interstitial water space in vivo. The technique can be applied in many ways. One way is to monitor the concentration of small molecules in a tissue and compare this with the concentration in blood to evaluate the contribution of that tissue to the metabolism of the molecule. Another way to use microdialysis is to add a drug or a hormone to the perfusion fluid, which does not cause generalized effects, and study the specific effect on that tissue. Focusing on methodological and physiological aspects, the general aim of this thesis was to investigate the possibility of using microdialysis in vivo, in human skeletal muscle, to study glucose metabolism, with special reference to insulin resistance and exercise. The specific aims were:

- I. to examine the use of a very low perfusion flow rate in microdialysis in order to directly measure interstitial concentrations of the metabolites glucose, lactate, glycerol and urea.
- II. to examine the effect (on metabolites and collected volume) of adding a colloid to the microdialysis perfusion solution (a colloid being necessary for avoiding fluid loss at a very low perfusion flow rate).
- III. to determine if it is possible to give insulin through the microdialysis catheter to study the local insulin sensitivity in human skeletal muscle by measuring the interstitial glucose concentration.
- IV. to determine the local effect of the insulin-mimetic compound vanadate on glucose metabolism in human skeletal muscle and examine if it is possible to use vanadate in combination with microdialysis as a measure of insulin resistance.
- V. to examine the local effect of insulin and vanadate on glucose metabolism in insulin resistant human skeletal muscle in the control state and after a single bout of exercise

MATERIALS & METHODS

SUBJECTS

Table 2.Characteristics of subjects participating in the experiments. Values are mean \pm SEM.

Study	Number of subjects	Age	Height (m)	Weight (kg)
I	18	24.9 \pm 0.9	180 \pm 1.9	79.7 \pm 2.6
II	13	24 \pm 2.8	182 \pm 0.02	77.7 \pm 2.7
III	13	29 \pm 2.7	183 \pm 1	77 \pm 2.0
IV	13	26.6 \pm 1.6	1.79 \pm 0.02	74.8 \pm 2.9
IV	5	54.2 \pm 7.9	1.86 \pm 0.03	118.8 \pm 6.3
V	8	51.5 \pm 2.1	1.78 \pm 0.02	100.5 \pm 5.76

MICRODIALYSIS

Catheters

All catheters used consisted of polyurethane tubing and a polyamide dialysis membrane. The catheters are needle-shaped with a double lumen cannula and a hollow fiber membrane at its tip (distal part). The molecular cut-off point for the dialysis membranes were 20 kDa in study I – V and also 100 kDa in study III – V. The length of the dialysis membrane was always 30 mm. The 20 kDa catheters used were CMA 60 (study I – V), figure 1, and the 100 kDa catheters were either CMA 60 (study III and IV) or CMA 71 (study V), all obtained from CMA Microdialysis AB, Stockholm, Sweden. In study III the microdialysis catheters were equipped with two inlet tubings.

Insertion of microdialysis catheters

Before insertion a local anaesthetic (0.3 ml of 1 % Carbocain, Astrazeneca, Södertälje, Sweden) was given subcutaneously and above the muscle fascia. Insertion of the catheters was performed using a steel-guide cannula or a guide tubing. In the steel guide procedure, the catheters were placed in a steel guide cannula when inserted; thereafter the steel guide cannula was instantly removed leaving the microdialysis catheter in place in the tissue. In the guide tubing procedure, a steel cannula with plastic tubing fitted on the outside was inserted in the tissue followed by immediate withdrawal of the steel cannula, leaving the tubing in place in the tissue. The microdialysis catheter was inserted in the tubing which then was removed by splicing upon retraction.

Perfusion fluid

The fluid used for perfusion of the catheters was a modified Krebs-Henseleit buffer (KHB). The bicarbonate was replaced with phosphate in order to achieve a stable pH in room temperature (Hickner, 1992). In all studies, except for half of the catheters in study II, the perfusion fluid was supplemented with dextran-70 in order to prevent fluid loss. In study III and V the perfusion fluid in some catheters were supplemented with

insulin and in study IV and V the fluid in some catheters were supplemented with vanadate.

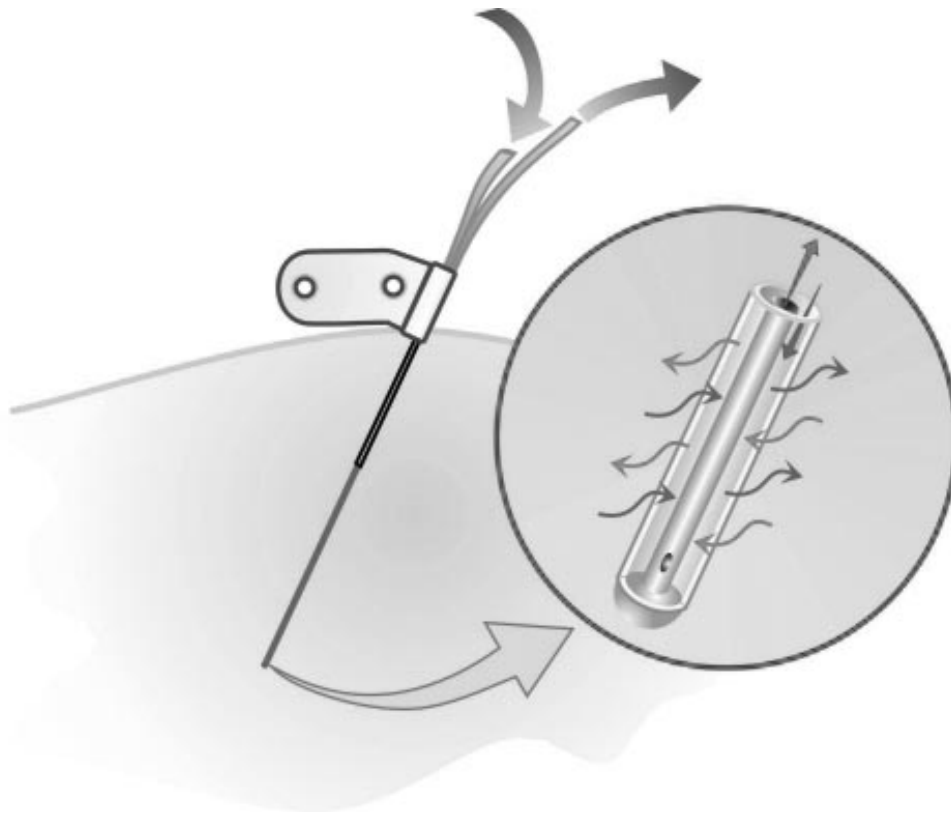


Figure 1. Microdialysis catheter (CMA 60). The catheter consist of a 30 mm long polyamide dialysis membrane (o.d. 0.52 mm), outer shaft tubing (polyurethane, o.d. 0.8 mm, length 20 mm), inlet tubing (polyurethane, o.d. 1.0 mm, i.d. 0.15 mm, length 400 mm), and outlet tubing (polyurethane, o.d. 1.0 mm, i.d. 0.15 mm, length 105 mm).

Perfusion flow rate

In all studies a very low perfusion flow rate was used in order to achieve complete equilibration between interstitial and perfusion fluids ($0.16 - 0.2 \mu\text{l}/\text{min}$). In study I a protocol with successively lower perfusion flows down to $0.075 \mu\text{l}/\text{min}$ were used in order to identify the perfusion flow rate where complete equilibration occurs. In study III and V a higher perfusion flow rate ($1.33 \mu\text{l}/\text{min}$) was used for the insulin catheters and their respective control catheters in order to prevent total adsorption of insulin in the plastic tubing and dialysis membrane. The pumps used were either CMA 100 or CMA 106 (CMA Microdialysis AB, Stockholm, Sweden)

BIOCHEMICAL ANALYSIS

Analysis of dialysate samples

The concentrations of glucose, lactate, glycerol, pyruvate and urea in the microdialysis samples were analyzed on CMA 600 Microdialysis Analyzer, using ordinary enzymatic

methods. The sample (0.2 – 0.5 µl) and reagent volumes (14.5 – 14.8 µl) are handled by a high-precision pipetting device. The rate of formation of the red-violet substance quinoneimine is measured in a filter photometer at 546 nm for glucose, lactate, glycerol and pyruvate. For urea, the rate of utilization of NADH is measured at 365 nm. By extracting the maximal absorbance change per second from the generated absorbance versus time curve, the instrument performs a kinetic measurement. Depending on the substance being measured, the complete measurement cycle takes between 60 and 120 s. The used reagents were obtained from CMA microdialysis and controls were included at regular intervals.

The concentration of insulin in the microdialysis samples was analyzed with a double-antibody radioimmunoassay (Pharmacia, Uppsala, Sweden).

The concentration of ethanol was determined using a modification of the procedure described by Bernt & Gutmann (Bernt, 1974). The analyses were made within one day of the experiment and are described more thoroughly in Rosdahl et al 1993 (Rosdahl, 1993).

Analysis of plasma samples

The plasma samples were analyzed using colorimetric methods on a Cobas Mira-s clinical analyzer (Roche Diagnostics, Basel, Switzerland). Earlier, verification between concentrations from the Cobas Mira-s analyzer and the CMA 600 analyzer was made to ensure that the concentrations are comparable (Rosdahl, 1998).

ORAL GLUCOSE TOLERANCE TEST

After an overnight fast the subjects in study V (and the insulin-resistant subjects in study IV) had a catheter inserted in an antecubital vein which was kept patent by a slow infusion of saline. The subjects were then given 75 g glucose in 250 – 300 ml water to drink over the course of five minutes. Blood samples were taken before the glucose load and at time 30, 60, 90 and 120 min after the glucose ingestion for plasma glucose and insulin measurements (WHO, 1999). The determination of blood glucose concentration were made using FreeStyle (TheraSense, USA). Subjects that had blood glucose concentrations (venous) above 6.7 mM (but below 10.0 mM) two hours after glucose ingestion (WHO, 1999) were included in the study. Subjects that according to the HOMA model (Homeostasis model assessment; $\text{fasting insulin } (\mu\text{U/ml}) \times \text{fasting glucose (mM)} / 22.5$) (Matthews, 1985) were insulin resistant (Nakai, 2002) were also included in the study.

STATISTICS

All values are given as means \pm SEM (standard error of mean) except for paper IV, where the values are given as medians \pm SD (standard deviation). This was due to the smaller number of subjects in each group in this study. The statistical significance was determined with Student's t-test for paired comparisons or one, two or three-way ANOVA with repeated measures design as indicated in the articles.

RESULTS

METABOLITE LEVELS IN HUMAN SKELETAL MUSCLE AND ADIPOSE TISSUE (STUDY I)

Experimental design

This study was designed to investigate if it was possible to identify a perfusion flow at which the interstitial fluid in human skeletal muscle and adipose tissue completely equilibrated with the microdialysis perfusion fluid. The metabolites studied were glucose, lactate, glycerol and urea. 18 male subjects had microdialysis catheters inserted in the vastus lateralis of the quadriceps femoris muscle and subcutaneous adipose tissue. Experiments with successively lowered perfusion flow rate were performed; 1.33 – 0.16 $\mu\text{l}/\text{min}$ as flow rates in 13 subjects and 0.33 – 0.075 $\mu\text{l}/\text{min}$ in five subjects.

Results

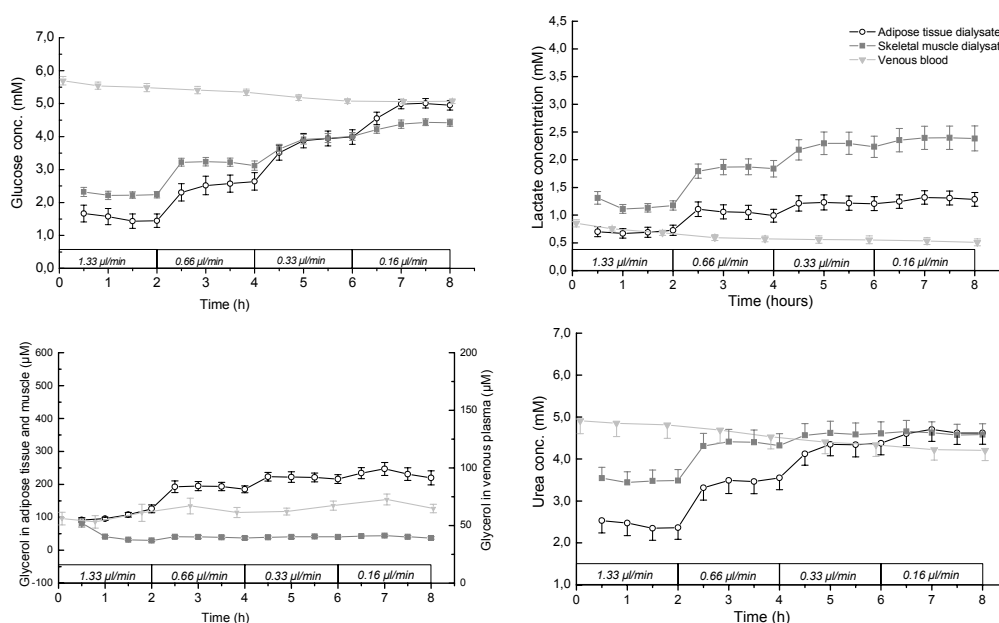


Figure 2. Glucose, lactate, glycerol and urea concentrations in venous plasma and microdialysis samples from skeletal muscle and adipose tissue with perfusion flow rates 1.33 – 0.16 $\mu\text{l}/\text{min}$.

A metabolite was considered completely equilibrated if the concentration did not increase when the perfusion flow rate was decreased. The glucose concentration was equilibrated completely at 0.16 $\mu\text{l}/\text{min}$ in both tissues, since the glucose concentration did not differ between 0.16 $\mu\text{l}/\text{min}$ and 0.075 $\mu\text{l}/\text{min}$. The lactate concentration was equilibrated at 0.33 $\mu\text{l}/\text{min}$, the glycerol concentration at 0.66 $\mu\text{l}/\text{min}$ in skeletal muscle and 0.33 $\mu\text{l}/\text{min}$ in adipose tissue and the urea concentration equilibrated at 0.33 $\mu\text{l}/\text{min}$ in skeletal muscle and at 0.16 $\mu\text{l}/\text{min}$ in adipose tissue.

Conclusion

We did show that it is possible to find a perfusion flow at which the interstitial fluid in human skeletal muscle and adipose tissue completely equilibrates with the microdialysis perfusion fluid. At complete equilibration, the measured concentrations of glucose, glycerol and urea were in good agreement with expected tissue levels. Overall, the study indicates that microdialysis at a low perfusion flow may be a tool for continuous monitoring of tissue interstitial concentrations.

EFFECTS OF ADDING A COLLOID TO THE MICRODIALYSIS PERFUSATE (STUDY II)

Experimental design

In this study we investigated the effect of changes in colloid osmotic pressure and in hydrostatic pressure of the perfusion solution on net fluid transport across the microdialysis membrane and on metabolite concentrations in the dialysate. Changes in the hydrostatic pressure were induced by varying the vertical position of the outflow tubing. Changes in osmotic pressure were induced by using perfusate with or without dextran-70. The experiments were performed both in vitro (the catheters placed in vials containing KHB with glucose, lactate, glycerol and urea) and in vivo (in the vastus lateralis of the quadriceps femoris muscle) in 13 male subjects.

Results

The sample volumes were significantly smaller in catheters perfused without a colloid compared with those perfused with a colloid. The sample volumes were also significantly smaller when the outflow tubing was influenced by maximal hydrostatic pressure (above position) compared with minimal hydrostatic pressure (below position). In vivo, glucose concentration at 0.33 $\mu\text{L}/\text{min}$ was higher when the catheters were perfused without a colloid than with a colloid. There was a tendency to a corresponding difference also in lactate, glycerol and urea. At 0.16 $\mu\text{L}/\text{min}$, the glucose concentration was the same irrespective of if fluid loss had been counteracted by colloid inclusion or by lowering of outlet tubing.

The mechanisms behind the difference in the glucose concentration at 0.33 $\mu\text{L}/\text{min}$ are thought to be 1) a higher effective perfusion flow rate when dextran-70 is added and 2) that the recovery of glucose is lower than for the other metabolites. Glucose is therefore more affected by changes in flow rate at flow rates where the concentrations in the interstitial and perfusion fluids are not yet equilibrated.

Conclusion

This study illustrates that both hydrostatic and osmotic pressures determine the net fluid transport across the dialysis membrane of a microdialysis catheter, both in vitro and in vivo in human skeletal muscle. Fluid balance could be achieved in this study by adding a colloid (dextran-70) to the perfusate or by maximally lowering an extended outflow tubing. This study shows that fluid imbalances can have important implications for microdialysis results at low perfusion flow rates.

INSULIN INDUCES LOCAL METABOLIC EFFECTS IN HUMAN SKELETAL MUSCLE WHEN PERFUSED THROUGH A MICRODIALYSIS CATHETER (STUDY III)

Experimental design

In this study the aims were 1) to examine the use of a large pore size dialysis membrane in human skeletal muscle 2) if dextran-70 could be used to prevent fluid loss with these membranes 3) if these membranes could be used to study interstitial concentrations of metabolites in skeletal muscle and 4) if it is possible to deliver insulin to the skeletal muscle and monitor its local effect on glucose transport.

Microdialysis catheters (CMA 60 with a 100 kDa mol. wt. cut-off dialysis membrane) were inserted in the quadriceps femoris muscle of 13 male subjects. In four subjects the catheters were perfused with successively lower flow rates (1.33 – 0.16 µl/min), in nine subjects the perfusion fluid to one catheter was supplemented with insulin at a successively increasing concentration.

Results

With different perfusion flow rates the recorded concentrations of glucose, lactate, glycerol and urea were in agreement with previously obtained values using a conventional membrane with a smaller pore size (20 kDa) (Study I).

When insulin was added to the perfusate the concentration of glucose was significantly reduced whereas no changes occurred in the control catheter.

Conclusion

This study shows 1) that the loss of perfusate from microdialysis catheters with a 100 kDa dialysis membrane can be prevented if a colloid is added to the perfusate and that this membrane is suitable for recordings of molecules in the interstitial space and 2) that large peptide molecules such as insulin diffuses across the dialysis membrane and has cellular effects that can be simultaneously recorded.

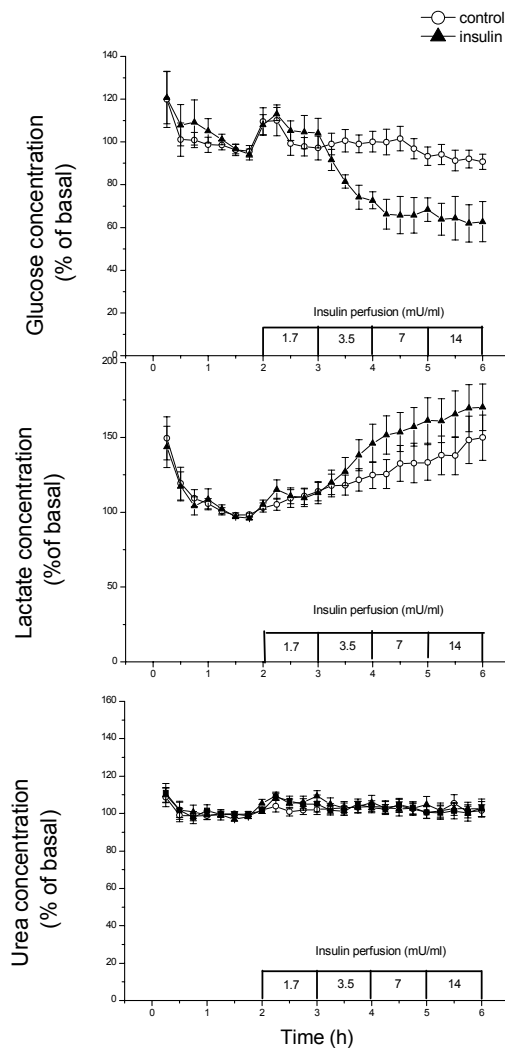


Figure 3. Glucose, lactate and urea concentrations presented as percent change from basal, in microdialysis samples from the insulin infusion experiment. Following a basal period, one catheter was perfused with a stepwise increased concentration of insulin while the other catheter served as control.

LOCAL EFFECT OF VANADATE ON INTERSTITIAL GLUCOSE AND LACTATE CONCENTRATIONS IN HUMAN SKELETAL MUSCLE (STUDY IV)

Experimental design

Study IV was undertaken to investigate the effect of the insulin-mimetic trace element sodium metavanadate on interstitial glucose and lactate concentrations in human skeletal muscle *in vivo*.

Interstitial concentrations of glucose and lactate were determined by microdialysis at a low flow rate in the quadriceps femoris muscle of 18 men. In the same leg two microdialysis catheters were inserted. In one catheter, the perfusion medium was

supplemented with sodium metavanadate (10-100 mM) after a basal period, the other catheter served as control.

Results

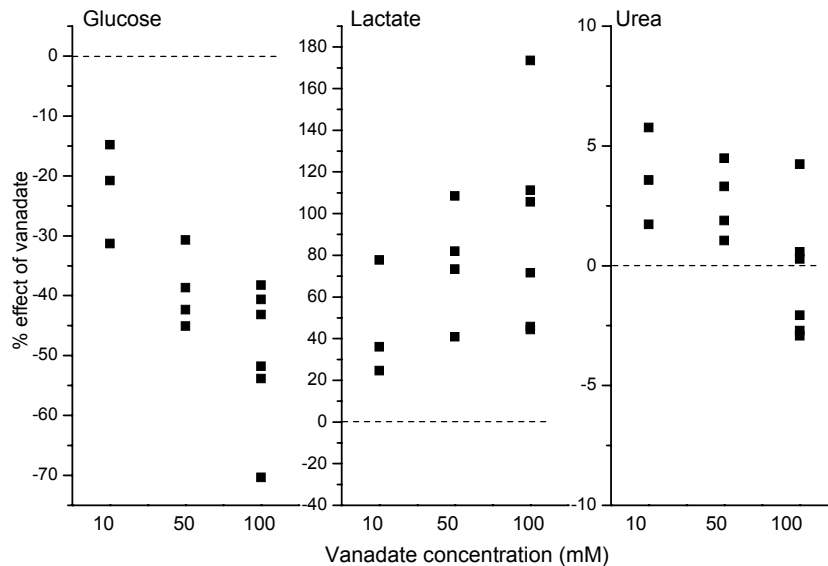


Figure 4. The effect (in percent) of perfusion with vanadate (10-100 mM) through microdialysis catheters placed in skeletal muscle of healthy human subjects on glucose (left), lactate (middle) and urea (right) concentrations in collected microdialysis samples. Each point represents one subject, with a comparison between one vanadate and one control catheter.

In the catheter perfused with metavanadate, the interstitial glucose concentration was decreased by 13 – 50 % compared to the control catheter ($p < 0.05$). The lactate concentration was higher in the 50 mM and 100 mM metavanadate catheters compared to control (39 – 89 %, $p < 0.05$). There was no difference between control and metavanadate catheters in urea concentrations. Five of the subjects were insulin-resistant and for them the results were similar, although the effect was somewhat smaller.

Conclusion

Perfusion with vanadate through microdialysis catheters in human skeletal muscle results in a decreased interstitial glucose concentration and an increased lactate concentration. This study thus indicates that vanadate mimics the effect of insulin in human skeletal muscle *in vivo*.

LOCAL EFFECT OF INSULIN AND VANADATE IN INSULIN-RESISTANT HUMAN SKELETAL MUSCLE: INFLUENCE OF A SINGLE BOUT OF ONE-LEGGED EXERCISE (STUDY V)

Experimental design

Study V was designed to test the hypothesis that a single bout of exercise augments the local effect of insulin and the insulin-mimetic trace element vanadate on the interstitial glucose concentration in insulin-resistant human skeletal muscle.

Eight insulin-resistant obese male subjects performed one bout (two hours) of one-legged cycle exercise. Microdialysis catheters were then inserted in the quadriceps femoris muscle in both legs. The perfusion medium was supplemented, after a basal period, with insulin (two catheters) or sodium metavanadate (two catheters). The other catheters served as control.

Results

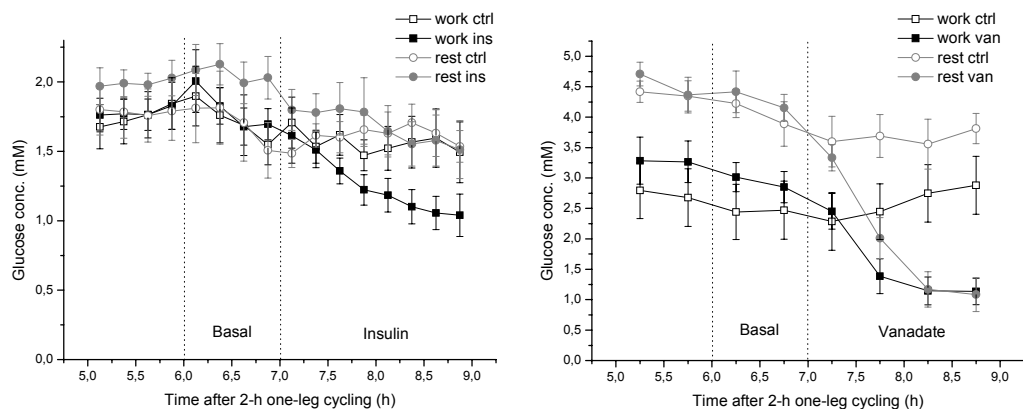


Figure 5. The glucose concentration from microdialysis catheters perfused with insulin (left), or vanadate (right). Open symbols are control catheters whereas filled symbols designate insulin/vanadate catheters, black line = working leg and grey line = resting leg.

In response to exercise the glucose concentration in the previously working muscle is decreased during several hours post exercise. This effect is above all seen in the catheters perfused with a low perfusion flow rate (Figure 5 right).

The effect of insulin on glucose concentration in the working leg (-32 %) is comparable with the effect seen in healthy muscle at rest (-30 %, Study III). The insulin effect in the resting (insulin-resistant) leg (-20 %) tended to be lower ($p < 0.09$) than the effect in the working leg.

This is quite the contrary to the effect seen after vanadate infusion, where the glucose concentration decreases to the same extent in both legs, and to the same or even higher than in healthy muscle (Study IV).

Conclusion

A single bout of exercise normalizes the insulin effect on the interstitial glucose concentration in insulin-resistant human skeletal muscle. The effect of vanadate (which is not decreased in insulin-resistant human skeletal muscle) was not augmented by exercise.

DISCUSSION

PERFUSION FLOW RATE

To obtain diffusion equilibrium between the interstitial and perfusion fluids a very low perfusion flow rate is a prerequisite. Study I was performed to find out how low the perfusion flow rate had to be to monitor the true interstitial concentrations of glucose, lactate, glycerol and urea. In skeletal muscle and adipose tissue, the perfusion flow rate for complete equilibrium for glucose concentrations was found to be 0.16 $\mu\text{l}/\text{min}$. For lactate the perfusion flow rate should be 0.33 $\mu\text{l}/\text{min}$ or lower, for glycerol and urea it should be 0.66 $\mu\text{l}/\text{min}$ for complete equilibration in skeletal muscle. In adipose tissue, the same values were obtained except for glycerol, where the perfusion flow rate had to be 0.16 $\mu\text{l}/\text{min}$ for complete equilibration.

Methodological variability

One of the advantages of using a very low perfusion flow rate is the low methodological variability calculated from the substance concentrations in adjacent microdialysis catheters inserted in the same tissue. In study I we observed that the variability for glucose and urea was reduced when the perfusion flow rate was lowered. We also observed that the methodological variation was higher in adipose tissue than in skeletal muscle. One of the reasons for the higher variability in the abdominal adipose tissue could be differences in skinfold thickness between subjects. In study I we found that the skinfold thickness was negatively correlated with recovery of glucose and urea. This could be due to reduced blood flow when the adipose tissue mass increases (Jansson, 1994).

INTERSTITIAL CONCENTRATIONS OF METABOLITES

Glucose

Glucose concentration in skeletal muscle at 0.16 $\mu\text{l}/\text{min}$, and at 0.075 $\mu\text{l}/\text{min}$, was found to be 0.6 mM lower than the concentration in venous plasma in study I. This result is comparable with studies using the no-net-flux method (Müller, 1996; Müller, 1995). In two other studies, where a perfusion flow rate of 0.3 $\mu\text{l}/\text{min}$ was used the recovery was incomplete (but very close to 100 %) (Moberg, 1997; Rooyackers, 2004) which is in agreement with our results since we had to lower the perfusion flow rate further from 0.33 $\mu\text{l}/\text{min}$ to obtain equilibrium.

Lactate

The lactate concentration found in skeletal muscle with microdialysis is higher in our study (study I) than in two previous studies based on the internal reference technique (Holmäng, 1998; Müller, 1996), where the lactate concentration was similar to that measured in human muscle biopsies (Lundberg, 2002). A higher lactate concentration in the interstitial fluid compared to the intracellular space is however expected because of a higher pH in the interstitial space (Henriksson, 1999). Other studies show as high lactate concentrations as in the present studies (Korth, 2000; Östman, 2004). One study showed a higher lactate concentration than in the present studies (Axelson, 2002) although using the same catheters and a perfusion flow rate of 0.3 $\mu\text{l}/\text{min}$.

Pyruvate

In study V we measured the pyruvate concentration in skeletal muscle for the first time. The achieved pyruvate concentration is higher than previously reported in studies performing microdialysis in human skeletal muscle (Axelson, 2002), (Östman, 2004). One possible reason for this could be the use of higher perfusion flow rates in the referred studies. The pyruvate concentration recorded is also higher than previously measured in muscle biopsies (Constantin-Teodosiu, 1999) and circulating levels (Berne, 1993). The reason for this discrepancy could be the higher pH in the interstitial space, which influences pyruvate as well as lactate (see above) (Henriksson, 1999).

Glycerol

The glycerol concentration in microdialysis samples from skeletal muscle is high initially which most likely is due to cellular damage caused by the insertion of the catheter. The glycerol concentration requires 90 – 120 min after catheter insertion to become stable (study I). This is in agreement with another study stating that the glycerol concentration should be back to baseline 90 – 150 min after insertion of the catheter (Gutierrez, 1999). The glycerol concentration in skeletal muscle in our studies are in agreement with other studies from the same muscle (Djurhuus, 2004; Hagström-Toft, 2002). It should be noted that the glycerol concentration differ between different muscle groups (Hagström-Toft, 2002).

Urea

The urea concentration in skeletal muscle in our studies is in agreement with other investigations (Östman, 2004). The measurement of the urea concentration was included since it provides information about the recovery and the diffusion condition of the tissue. Some investigators have attempted to use urea as a reference compound for in vivo calibration of the recovery of microdialysis catheters (Brunner, 2000; Ettinger, 2001).

PERFUSATE LOSS

At very low perfusion flow rates loss of fluid is a problem that must be solved in order to use the technique. Both hydrostatic and osmotic pressures determine the net fluid transport across the dialysis membrane and therefore measures to change both hydrostatic and osmotic pressures can be applied to create fluid balance across the microdialysis membrane. In this thesis the fluid loss was counteracted by adding a colloid (dextran-70) to the perfusion solution in all studies. Another approach to counteract fluid loss is to use a “push-pull” pumping system (Sjögren, 2002) instead of the ordinary push pumps (like CMA 100) or to apply suction to the outflow tubing (Hoffner, 2003). However, in spite of the inclusion of a colloid, fluid imbalances can influence the metabolite concentrations, as was shown in study II. To carefully control for fluid imbalances we always weigh the microvial before and after sample collection.

INFUSION THROUGH THE MICRODIALYSIS CATHETER

By perfusion with different hormones/drugs through the catheter, the microdialysis technique allows direct measurement of effects on metabolite concentrations thus avoiding generalized effects that can be seen in metabolic infusion studies.

Insulin

Both in study III and V we infused insulin through the microdialysis catheter in human skeletal muscle and recorded a decreased glucose concentration which is assumed to reflect a higher rate of glucose uptake. To our knowledge this procedure has only been used once previously (MacLean, 2001) showing the same results.

Before study III we performed pilot experiments where we added insulin to the perfusion fluid and used a low flow rate (0.2 $\mu\text{l}/\text{min}$) without seeing any effect on the glucose concentration (unpublished results). This could be due to higher adsorption of insulin to the tubing when the flow rate is lowered (Hewson, 2000). This is the reason why the insulin catheters in study III and V are perfused with a higher flow rate (1.33 $\mu\text{l}/\text{min}$). The adsorption of insulin was measured in study III where the actual insulin concentration in the syringe (connected to the inflow tubing of the microdialysis catheter) was 14 mU/ml. The insulin concentration in the dialysate was then found to be 3.2 mU/ml.

We did not measure the recovery in these studies, but a previous study, measuring interstitial insulin concentration in human skeletal muscle reported a recovery of 3 %, using the same catheters and similar (1 $\mu\text{l}/\text{min}$) perfusion flow rate (Sjöstrand, 1999). Taking this value and the observed adsorption of insulin (to the syringe, plastic tubing and dialysis membrane) into account the tissue immediately surrounding the catheter was estimated to be influenced by an insulin concentration of around 90 $\mu\text{U}/\text{ml}$ in both study III and V. The study by MacLean et al perfusing with insulin showed a significant glucose uptake (measured as the difference between recovery of D-glucose and L-glucose) at 1.0 $\mu\text{U}/\text{ml}$ with no further increase at 10 $\mu\text{U}/\text{ml}$. However, these authors did not report any adsorption of insulin (MacLean, 2001). 90 $\mu\text{U}/\text{ml}$ insulin concentrations in serum are often seen during studies with the euglycemic hyperinsulinemic clamp technique (Rosdahl, 1998).

One could argue that insulin causes an increased blood flow, although this is still under debate (Clark, 2003). If the blood flow was increased due to the insulin infusion the glucose concentration would rise, therefore resulting in an underestimation of the cellular effect of insulin. However, the present results (Study V) using the microdialysis ethanol technique indicates that insulin does not cause a change in nutritive blood flow when added to the perfusate.

Vanadate

In study IV and V sodium metavanadate was infused through the microdialysis catheter in human skeletal muscle. The results clearly show that metavanadate mimics the effect of insulin. This is true also in insulin-resistant skeletal muscle where the effect does not seem to be decreased relative to healthy insulin-sensitive muscle. In fact, using 100 mM sodium metavanadate, the effect on the interstitial glucose concentration was higher in the insulin-resistant subjects (Study V: -66 % in the exercised leg and -71 % in the rested leg) than in the healthy subjects (Study IV, no exercise had been

performed: 47%). The corresponding values for the interstitial lactate concentration were 61 % and 43 % (exercised and rested leg, respectively) in insulin-resistant muscle and 89 % in healthy muscle. A possible explanation for this different results could be systemic effect following the exercise on the glucose homeostasis (Ahlborg, 1975) That vanadate induces glucose lowering in insulin-resistant states is in agreement with other studies (Goldfine, 1995; McNeill, 1994; Shechter, 1990).

After oral treatment in type-2 diabetic patients, 50% of the patients (4 out of 8) improved their insulin sensitivity (Willsky, 2001). In another study with oral treatment in healthy human subjects no effect of vanadyl sulphate on insulin sensitivity, fasting plasma glucose and insulin levels was found (Jentjens, 2002).

The precise mechanism of action of vanadate on tissue glucose uptake has been difficult to reveal (reviewed in (Cam, 2000). The reasons for this could be that there are different kinds of vanadium salts used and also that the effect of different concentrations of the compound have been reported in different studies. The reason for choosing the metavanadate in the present studies was that a solution of metavanadate has a neutral pH (Elvingson, 1997).

Earlier studies have shown a maximal rate of glucose transport at 30 mM vanadate in muscle samples (*in vitro*) from morbidly obese patients (Carey, 1995). The results from Study IV are in general agreement with this finding, since we observed a larger effect with 50 than with 10 mM of sodium metavanadate, whereas the effect was not further increased when 100 mM sodium metavanadate was used. The concentrations of vanadate used in the present studies are however markedly higher than what is generally observed in the blood after oral treatment (Willsky, 2001). Therefore it is possible that the observed therapeutic effect of vanadate in insulin-resistant states is related to other effects than an increased glucose uptake in skeletal muscle.

GLUCOSE TOLERANCE

In study IV and V we performed oral glucose tolerance tests to identify individuals with an insulin-resistant state. Since obesity decreases glucose tolerance by progressively increasing the insulin resistance (Carlsson, 1998), we chose to test obese subjects. In the Stockholm Diabetes Prevention program 12.5 % of the population with a BMI above 28 kg/m² and 6.9 % of the population with a family history of diabetes have impaired glucose tolerance (Carlsson, 1998). The insulin resistance also increases with increasing age; in the above mentioned program 4.4 % of the population between 35-45 years of age and 6.2 % of the population between 46-56 years of age had impaired glucose tolerance (0.8 % and 2.4 % had type-2 diabetes, respectively). With this in mind we tested obese subjects in the older age group (51.5 ±2.1 yr), preferable with a family history of diabetes. Out of 23 subjects tested prior to study V, eight were shown to have impaired glucose tolerance (blood glucose concentration above 6.7 mM but below 10 mM two hours after glucose ingestion) or a HOMA well above 2.77 (Nakai, 2002).

PHYSICAL ACTIVITY AND INSULIN SENSITIVITY

In study V, the obese insulin-resistant subjects exercised with one leg for two hours in order to increase the insulin-sensitivity of one leg, the other leg serving as control. The

insulin-sensitivity should be increased after two hours since other investigators have reported enhanced insulin sensitivity in the working leg of healthy individuals after one hour of one-legged exercise at varying intensity (measuring glycogen synthase activity in muscle biopsies before and after a hyperinsulinemic-euglycemic clamp) (Wojtaszewski, 2000) as well as increased insulin sensitivity following 30 min of high intensity exercise (hyperinsulinemic-euglycemic clamps and measuring glycogen synthase activity in muscle biopsies) (Devlin, 1985). However, 30 min of one-legged exercise at moderate intensity did not increase insulin sensitivity in healthy or type-2 diabetic subjects (measuring glycogen synthase activity in muscle biopsies before and after hyperinsulinemic-euglycemic clamps) (Dela, 1995).

The increase in insulin sensitivity (measured with biopsies and hyperinsulinemic-euglycemic clamps) after one bout of exercise may last for as long as 48 hours (Mikines, 1988). A previous microdialysis study shows that a 2 h exercise bout leads to a prolonged decrease in muscle interstitial glucose concentration. This decrease may last for 6-7 hours or more (Henriksson and Knol to be published in *Acta Physiologica Scandinavica*). To avoid these acute effects of exercise the microdialysis measurements were performed six hours after the exercise bout in Study V.

The glucose concentration at basal, from the low-flow catheters was decreased in the working leg compared to the resting leg indicating an acute effect of exercise still present. This result is not seen in the high flow catheters, though. We have previously seen that the methodological variability for glucose and urea was lower when the perfusion flow rate was decreased (Study I). This higher methodological variability could be the reason why the lower glucose concentration is not detected in the high-flow catheters.

Part of the lowering of the glucose concentration in the working leg could however also represent the higher insulin sensitivity since the present results showed that the lowered effect of insulin on the interstitial glucose concentration in insulin-resistant muscle was normalized following the 2-hour bout of exercise. A previous study has indicated that the arterial-interstitial (a-i) difference in glucose concentration can be used as a measure of insulin sensitivity (Holmång, 1998), as no arterial-interstitial (a-i) differences were found in insulin-resistant skeletal muscle but were present in insulin sensitive human skeletal muscle. Taken together, the available data indicate that the microdialysis technique could be used to measure the degree of insulin resistance in human skeletal muscle both from insulin infusion experiments and from measurements of the arterial-interstitial (a-i) glucose difference.

CONCLUSIONS

The microdialysis technique has been shown to be useful in many ways since it was first introduced in human skeletal muscle. In this thesis the main focus has been to evaluate the technique of using very low perfusion flows in order to obtain equilibrium between interstitial and perfusion fluids and to use this technique to study glucose metabolism and insulin resistance in human skeletal muscle.

We found that to obtain complete equilibration between the interstitial and perfusion fluids in human skeletal muscle, a perfusion flow rate as low as 0.16 $\mu\text{l}/\text{min}$ is necessary with the presently used catheters (Study I).

At these low perfusion flow rates a colloid needs to be included in the perfusion fluid in order to keep the fluid balance and in this thesis it was shown that the colloid dextran-70 does not affect the metabolite concentrations in the collected dialysates (Study II).

A high cut-off microdialysis membrane can be used to infuse insulin into human skeletal muscle and record its effect on metabolite concentrations (Study III).

When the insulin-mimetic agent vanadate was infused via the microdialysis catheter into human skeletal muscle (Study IV), it was found to decrease the interstitial glucose concentration in a similar way as insulin, but in contrast to insulin (see below), the effect of vanadate was not diminished in insulin-resistant skeletal muscle and was not affected by exercise.

Following one-leg cycling for two hours, the effect of insulin in insulin-resistant subjects tended to be higher in the working compared to the resting leg. The effect in the working leg was similar to the effect in healthy resting muscle seen in study III. Therefore it is concluded that a single bout of exercise normalizes the insulin effect on the interstitial glucose concentration in insulin-resistant human skeletal muscle. This is contrary to the effect seen after vanadate infusion, see above.

POPULÄRVETENSKAPLIG SAMMANFATTNING

NY TEKNIK FÖR ATT STUDERA INSULINRESISTENS I

SKELETTMUSKULATUR.

Insulinresistens (sänkt insulinkänslighet) är ett känt riskfaktorsyndrom för hjärtkärlsjukdom, även kallat det metabola syndromet. Skelettmuskulaturen är den vävnad som till största delen svarar för insulinresistensen. Insulinresistensen kan vid ärftlig predisposition leda till typ-2 diabetes ("åldersdiabetes").

Känsligheten för insulin ökar med ökad fysisk aktivitet. Det betyder att man får en ökad insulinkänslighet i de muskler som har varit aktiva, men inte i de muskler som varit inaktiva. Ökningen i insulinkänslighet är troligen en av förklaringarna till varför fysisk träning är en av de bästa metoderna för att minska risken för att dö en alltför tidig död i hjärt-kärlsjukdom. Tillgängliga data antyder dessutom att en betydande effekt kan uppnås med en ganska beskedlig dos fysisk aktivitet. Mekanismerna bakom de positiva effekterna av en ökad fysisk aktivitet är idag inte helt kända.

Det finns ett antal olika metoder för att mäta graden av insulinresistens hos en individ. Gemensamt för dessa är att de framförallt mäter insulinresistens i hela kroppen. Många gånger skulle det räcka med att enbart titta på skelettmuskulaturen eftersom insulinresistensen ofta syns först där.

Mikrodialys är en metod som gör det möjligt att kontinuerligt följa en vävnads ämnesomsättning. En mikrodialyskateter är en tunn slang tjock som en fin injektionsnål. Den kan liknas vid ett konstgjort blodkärl (kapillär), som förs in i den vävnad man vill studera (i vårt fall human skelettmuskulatur). Katetern består bl a av ett semipermeabelt membran och en fysiologisk buffertlösning spolats långsamt igenom katetern. Kemiska substanser diffunderar till och från mikrodialyskatetern på samma sätt som ämnen diffunderar till och från en kapillär. Vätskan som har spolats igenom katetern innehåller olika substanser, t ex glukos, i samma koncentration som i muskelns vävnadsvätska. Mikrodialysen ger därmed ett mer vävnadsspecifikt värde än ett blodprov som representerar ett medelvärde av alla kroppens organ.

Först utvecklade vi mikrodialys tekniken för att kunna använda den på ett enkelt sätt i skelettmuskulaturen. Genom att genomföra en undersökning där vi successivt sänkte flödes hastigheten för vätskan som spolades genom mikrodialyskatetern kom vi fram till vilken flödes hastighet som måste användas för att se sanna koncentrationer av olika ämnen i vävnadsvätskan. Om man vid mikrodialys använder en hög flödes hastighet så hinner inte diffusionen med att skapa en jämvikt mellan vävnadsvätskan och mikrodialyskatetern – det blir en lägre koncentration av olika ämnen i mikrodialyskatetern än i vävnadsvätskan. I undersökningar där man använder en hög flödes hastighet måste man genomföra kalibrering av mikrodialyskatetern för att få fram den sanna koncentrationen av ett ämne i vävnadsvätskan.

Vi insåg att vid de låga flödes hastigheter som vi ville använda så försvann en del av perfusionsvätskan (den vätska som spolats igenom mikrodialyskatetern) ut i vävnaden. För att motverka detta tillsattes en kolloid i vätskan, dvs. en molekyl som är för stor för

att kunna diffundera ut i vävnaden och som därför håller kvar vätskan i mikrodialyskatetern (bildar sk kolloidosmotiskt tryck). I arbete II genomförde vi en undersökning där vi visade att denna kolloid (dextran-70) inte påverkade resultaten av mikrodialysen i övrigt.

Mikrodialys tekniken användes sedan för att se om det var möjligt att studera effekter av hormonet insulin i skelettmuskulatur genom att tillsätta det i perfusionsvätskan. Insulin är det hormon i kroppen som får musklerna att ta upp glukos (socker) från blodet och därmed sänka blodsockernivån. När insulin tillsattes i perfusionsvätskan kunde vi se att insulinet diffunderade ut till muskeln eftersom skelettmuskulaturen svarade genom att ta upp mer socker (glukoskoncentrationen i mikrodialyskatetern sänktes).

Vi fortsatte därefter med att tillsätta vanadat i perfusionsvätskan. Vanadat är ett ämne som har liknande effekter som insulin. I mindre undersökningar i USA har vanadat testats som ett möjligt läkemedel till patienter med typ-2 diabetes. Man har då sett att vanadat sänker blodsockernivån i blodet.

När vanadat fanns med perfusionsvätskan sänktes glukoskoncentrationen i mikrodialyskatetern – samma resultat som när vi tillsatte insulin. I denna undersökning inkluderade vi även några äldre insulinresistenta individer som också visade på samma resultat. Kanske går vanadat ”förbi” deras insulinresistens.

I den sista undersökningen tillsatte vi antingen insulin eller vanadat i mikrodialyskatetrarna (insulin i två och vanadat i två). Försökspersonerna var alla insulinresistenta men fick före undersökningen cykla med ena benet på en motionscykel i två timmar.

Efter en-bens cyklingen hade försökspersonerna ett ben där musklerna var insulinresistenta (vilobenet) och ett ben som var insulinkänsligt (arbetsbenet).

Mikrodialysundersökningen visade att insulin hade bättre effekt på glukoskoncentrationen i arbetsbenet än i vilobenet, medan vanadat hade lika bra effekt i båda benen. Effekten av insulin i arbetsbenet var lika stor som insulineffekten hos de friska individer studerades i arbete III. Slutsatsen är att endast ett arbetspass kan normalisera skelettmuskelns insulinkänslighet.

Arbetet stöder också teorin att effekten av vanadat inte påverkades av insulinresistensen. Vanadateffekten var varken sänkt hos dessa insulinresistenta individer i jämförelse med de friska individerna i arbete IV eller förändrades av arbete.

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